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In Vitro Antiviral Activity of Black Tea Polyphenols on Sindbis Virus in Vero Cells

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Abstract

Black tea, derived from the *Camellia sinensis* plant, is a natural product that is rich in polyphenols. The major polyphenols found in black tea are known as theaflavins. Previous research demonstrated that theaflavins contain antiviral activity against Herpes simplex virus 1 & 2 (HSV-1 & HSV-2), both enveloped DNA viruses. The goal of this research was to investigate the antiviral activity of theaflavins on the Sindbis virus (SINV). SINV is an enveloped RNA virus, and the prototype species of alphaviruses. SINV was treated with various concentrations of a theaflavin rich Black Tea Extract (BTE) before infection of Vero Cells. Antiviral and ToxGlo assays indicate that BTE exhibits antiviral activity against SINV at concentrations of 0.7mM and 1.4mM. Treatment of virus with 1.4mM BTE inhibits viral infection by 99%, while 0.7mM BTE inhibits viral infection by 46 - 74%. Cytopathic effect images suggest that 1.4mM BTE reduces cytopathic effects, correlating with the antiviral and ToxGlo assay results. Altogether, this research indicates that BTE does inhibit Alphavirus replication *in vitro*. The inhibition of SINV replication in this study would suggest potential inhibition of other Alphaviruses.

MONTCLAIR STATE UNIVERSITY

In Vitro Antiviral Activity of Black Tea Polyphenols on Sindbis Virus in Vero Cells

by

Jose Villagomez

A Master's Thesis Submitted to the Faculty of

Montclair State University

In Partial Fulfillment of the Requirements

For the Degree of Master of Science

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Introduction

Tea, derived from the *Camellia sinensis* plant, has historically been one of the most consumed beverages in the world. Most of its biological activity and medicinal values can be attributed to polyphenols. Tea polyphenols have recently been the center of attention due to their anti-inflammatory, anticancer, antioxidative, antibacterial, and antiviral properties (Zu et al., 2012; Bedran et al., 2015; Datta et al., 2014; Konarikova et al., 2015). Theaflavins are the most abundant polyphenols found in black tea. The four theaflavins found in black tea are theaflavin (TF1), theaflavin-3-monogallate (TF2A), theaflavin-3' –monogallate (TF2B), and theaflavin-3-3'-digallate (TF3). The antiviral properties of theaflavins are of interest to our lab. Black Tea Extract (BTE; $\geq 80\%$ theaflavin), a crude theaflavin extract is not only non-cytotoxic in Vero cells (0.014 μ M-14mM), but also exhibits antiviral activity against HSV-1 and 2 (Cantatore et al., 2013). Antiviral activity of theaflavins against HIV-1, bovine coronavirus and rotavirus has also been reported (Clark et al, 1998; Yang et al, 2012). It has been suggested that theaflavins interfere with viral glycoproteins, preventing adsorption, and ultimately viral infection (de Oliveira et al., 2015).

Sindbis (SINV) is an Alphavirus that contains a positive sense single stranded RNA genome enclosed by an icosahedral nucleocapsid that is surrounded by a host derived lipid bilayer embedded with glycoproteins. Transmitted by the Culex mosquito species, SINV infections have been reported in Africa, Finland, Sweden, and Russia. Infections lead to fever, fatigue, rash, and polyarthrititis that may remain for up a year post infection. SINV is an Old World Alphavirus, not traditionally found in the Americas, and as of today there have been no reported cases of SINV in the new world.

On the other hand, cases of Chikungunya, a more pathogenic Old World Alphavirus, have been reported in the Caribbean and United States for the first time. (Leparc et al, 2014; WHO 2016).

SINV acts as the model Alphavirus in laboratories, due to its low pathogenicity. At the moment no approved vaccine or antiviral drug exists to treat Alphavirus infections. Therefore, the discovery of novel antiviral molecules is of great interest. The abundance and cost efficiency of theaflavins from black tea make them ideal candidates to be studied as novel antiviral treatments. In this study, SINV was pretreated with 0.7mM and 1.4mM BTE prior to infection of Vero cells, in order to evaluate the antiviral activity of black tea theaflavins. The inhibition of SINV replication in this study would suggest potential inhibition of other Alphaviruses.

Review of Literature

Sindbis (SINV) is a positive sense single strand RNA virus. The virus was first isolated from the *Culex pipiens* and *Culex univittatus* mosquitoes in 1952, in a village a few kilometers outside of Cairo, Egypt (Taylor et al., 1955). SINV contains an 11.5kb single stranded RNA genome. The genetic material is enclosed in a protein capsid, forming an icosahedral nucleocapsid (NC). The NC is surrounded by a host-derived lipid bilayer envelope embedded with viral glycoproteins (Jose et al., 2009). The genome is composed of two open reading frames, encoding four non-structural (nsP1-4) and five structural proteins (C, E3, E2, 6K, and E1) (Supp. Figure 1). The alphavirus life cycle is shown in supplemental figure 2 (Schwartz and Albert 2010). The E2 structural protein has been associated with cell receptor recognition, allowing for entry to the host cell (Smith et al., 1995). Studies indicate that E2 interacts with the host cell surface glycosaminoglycan heparan sulfate during adsorption (Klimstra et al., 1998 and Smit et al., 2002). Although virus binding is reduced in heparan sulfate deficient cells, SINV is still able to infect and replicate, suggesting that other cell receptors are involved in viral entry (Byrnes et al., 1998). As virions enter the host cell via receptor mediated endocytosis, they reside in endosomes. The acidic environment in endosomes results in exposure of the E1 protein, which allows for host and viral membrane fusion. This results in the release of the viral genome. Non-structural proteins are translated, and a replication complex composed of nsP1-4 is formed. nsP1 is responsible for the synthesis of the minus strand viral RNA (Salonen et al., 2005). The nsP2 has RNA helicase properties, and is involved in shutting down host cell transcription (Cedron et al., 1999). nsP3 is included in the replicase unit, and nsP4 acts as the viral RNA polymerase (Leung et al.,

2011). The replication complex synthesizes a negative strand RNA intermediate that functions as a template for synthesis of genomic and subgenomic RNA. Structural proteins are expressed as the polyprotein C-pE2-6K-E1. Viral assembly begins by RNA packaging, nucleocapsid assembly, and processing of glycoproteins that are transported to the plasma membrane. Newly formed alphavirus virions bud off at the cell membrane (Schwartz and Albert 2010).

SINV belongs to the *Alphavirus* genus, and the family *Togaviridae*. Some other Alphaviruses are Chikungunya virus, Semliki Forest Virus, Western equine encephalitis virus, Eastern equine encephalitis virus, and Venezuelan equine encephalitis virus (Jose et al., 2008). These viruses are categorized into Old World and New World viruses. SINV is an Old World Alphavirus, along with Chikungunya virus and Semliki Forest Virus. Like most Alphaviruses, SINV is transmitted via mosquito vectors. SINV is able to infect both non-vertebrate (mosquitos) and vertebrate hosts (humans and birds) (Kurkela et al., 2008). Host cells susceptible to SINV infection include striate and smooth muscle cells, neuron and glial cells, and synovial cells (Strauss et al., 1994, Niekerk et al., 2014). Scientists have gained an interest in using SINV as a viral vector in gene therapy due to its diverse tropism and high transient gene expression (Lundstrom., 2009). SINV's ability to infect neural cells makes it an ideal candidate to be used to treat central nervous system diseases.

SINV serves as the model Alphavirus in labs worldwide, due to its low pathogenicity compared to other Alphaviruses. Infections in humans have been reported in Africa as O'nyong-nyong disease, and in Fennoscandia as Pogosta (Finland), Ockelbo (Sweden), and Karelian fever (Russia) (Laine et al., 2004; Trunen et al., 1998; Kurkela et

al., 2008). Symptoms include fever, fatigue, papular rash, and joint inflammation. The incubation time of the infection is 2 to 10 days. Although most symptoms disappear, joint pain may remain for up to a year post infection. Previous studies show that chronic arthritis may develop after a SINV infection (Laine et al., 2004; Luukkainen et al., 2000).

SINV is able to infect non-human vertebrates such as birds. In a study conducted in Finland, SINV was detected in both resident and migratory birds (Kurkela et al., 2008). Infection of migratory birds may give rise to the spread of SINV in other areas of Europe, and potentially the New World. In fact, this phenomenon has already been reported for the Chikungunya virus, a close relative to SINV. The first Chikungunya infection in the New World was reported in the Caribbean in 2013, leading to a major outbreak of the virus (Leparc et al., 2014). Furthermore, earlier this year the first locally acquired case of Chikungunya infection in the United States was reported in Texas (WHO., 2016). As temperatures rise, so do mosquito populations, increasing the probability Chikungunya cases spreading throughout the United States. Therefore, it's important to identify novel molecules that can be used as drugs to treat Alphavirus infections.

Alleviating Alphavirus associated symptoms is the only treatment for these types of infections. Chloroquine has been reported as an effective treatment of chronic arthritis associated with Alphavirus infections (Brighton 1984). On the other hand, a clinical study including 27 Chikungunya infected patients showed no significant difference in arthralgia and viremia between the placebo and chloroquine treated groups (De Lambellerie et al., 2008). More recently, a glycosaminoglycan-like molecule pentosane polysulfate, has been shown to alleviate virus-induced arthritis (Herrero et al., 2015). This molecule only treats the symptoms associated with viral infection, but does not prevent viral replication.

Inhibiting viral infection and decreasing viremia is a more effective treatment option for Alphavirus infections. Arbidol, a broad spectrum antiviral used for respiratory viral infections, was found to have antiviral activity against Chikungunya *in vitro* (Delogu et al., 2011). The anti-parasitic drug suramin was also found to inhibit Alphavirus replication by interfering with RNA synthesis and earlier steps in replication (Albulesco et al., 2015). As of today, no approved antiviral drug or vaccine for SINV and other Alphaviruses exists. Therefore, the discovery of a novel antiviral molecule is of great interest and importance.

Tea is one of the most consumed beverages in the world. Black tea in particular makes up almost 80% of all tea leaves manufactured worldwide (Kuroda and Hara., 1999). The *Camellia sinensis* plant is the source of green tea, oolong tea, and black tea. Polyphenolic compounds known as flavonoids make up a large portion of the chemical composition of these teas. The major flavonol of green tea is the catechin, representing 80% of all polyphenols found in green tea (Shahidi et al., 1999). Fermentation of *Camellia sinensis* tea leaves gives rise to black tea. During the fermentation process, catechins undergo a series of oxidative reactions, resulting in the synthesis of the major black tea flavanols called theaflavin and thearubigins (Shahidi et al., 1999). The major theaflavins found in black tea are theaflavin (TF1), theaflavin-3-monogallate (TF2A), theaflavin-3'-monogallate (TF2B), theaflavin-3,3'-digallate (TF3) (Li et al, 2013). Parallel to green tea catechins, black tea theaflavins are recognized for having anti oxidative, anti-inflammatory, anticancer, antibacterial, anti-arthritic and antiviral properties (Zu et al., 2012; Bedran et al., 2015; Datta et al., 2014; Konarikova et al., 2015). Cantatore et al. (2013) determined that treatment of herpes simplex virus -1 (HSV-

1) virions with BTE significantly reduced viral titers. Black tea antiviral effects against Alphaviruses have yet to be studied, and is of great interest to our lab.

Although crude black tea extracts have been shown to have antiviral properties, TF3 alone is able to inhibit HSV-1 and influenza infectivity (Oliveira et al., 2015; Nakayama et al., 1993; Zu et al., 2012). Theaflavin antiviral activity against HIV-1, bovine coronavirus, and bovine rotavirus has also been reported (Clark et al., 1998; Yang et al., 2012). A recent study revealed anti-Chikungunya activity of epigallocatechin gallate (EGCG), the major polyphenol of green tea (Weber et al., 2015). Since black tea theaflavins are derived from green tea catechins, it can be hypothesized that black tea also possesses anti-Alphavirus activity. The use of SINV, the prototype Alphavirus, in our studies will provide insight on the broad spectrum antiviral activity of black tea polyphenols. Mode of action studies are also of interest when investigating black tea extract activity.

Previous studies suggest that black tea theaflavins inhibit viral infection by interacting with viral glycoproteins, disrupting early steps in the viral cycle (Cantatore et al., 2013; Zu et al., 2012). A well-known characteristic of Alphaviruses is their ability to shutdown host cell transcription and translation (Akhrymuk et al., 2015). Taking over the host cell prevents an immune response from the host cell, leading to effective viral replication. Furthermore, during SINV genome replication, dsRNA intermediates are synthesized. To prevent detection of dsRNA by the host cell, SINV encloses its RNA in structures called membrane spherules (Frovolá et al., 2010). The use of spherules by Alphaviruses not only makes it difficult for the host cell to detect a viral RNA, but also causes difficulty when designing drugs targeting viral RNA degradation due to inefficient

drug delivery. Theaflavins possess inhibitory effects on hemagglutinin found on the surface of the Influenza virus (Zu et al., 2012). A study using monoclonal antibodies to inhibit Alphavirus infections revealed that these antibodies bind to the B domain of the E2 glycoprotein, inhibiting attachment of the virus to the host cell (Fox et al., 2015). The E2 glycoprotein may be a target of theaflavins, although this has not been confirmed.

The diverse tropism of SINV suggests that cell receptors and virus-host cell interactions may be conserved among all susceptible tissue. Therefore, the discovery of molecules targeting initial virus-host cell interactions, like black tea polyphenols, is advantageous. A molecule targeting viral entry may hold the potential to serve as an antiviral drug for a broad spectrum of viruses.

Methods

- **Cell culture:**

Vero cells were maintained in T25 flasks at 37°C and 5% CO₂ in Dulbecco's Modified Eagle Media (DMEM) supplemented with 5% Fetal Bovine Serum (FBS) and 1 mg/mL gentamicin. 0.25% Trypsin EDTA was used to subculture cells.

- **Preparation of Black Tea Extract:**

Black Tea Extract (BTE) \geq 80% theaflavin (10mg) (Sigma-Aldrich, Saint Louis, MO, USA) was dissolved in 1 ml of DMEM to prepare a BTE stock solution of 14mM. Concentrations of 1.4mM and 0.7mM were prepared by dilutions with Dulbecco's Modified Eagle Media.

- **Virus :**

Stocks of Sindbis virus were maintained for the experiments. A monolayer of cells was infected, and incubated until complete cytopathic effects (CPE) were present. Media was collected and centrifuged to separate cellular debris. The supernatant was transferred to cryotubes and stored at -80°C for use in experiments.

- **Viral titer determination using plaque assay**

Vero cells were seeded in 6 well-plates, and allowed to proliferate to 100% confluency. Ten-fold serial dilutions of SINV were prepared prior to infection. Vero cells were then infected with 500 μ L of each dilution prepared, and allowed to adsorb for 1h at 37°C and 5% CO₂. Unabsorbed viruses were removed, and plates were overlaid with nutrient medium-containing agar. The cells were

incubated for 5 days at 37°C and 5% CO₂. Cells were visualized by staining with crystal violet. The plaque assay was done in duplicates. Titer was used to determine the multiplicity of infection (MOI).

- **Cytopathic Effect Images:**

Vero cells were seeded onto 60mm dishes, and allowed to proliferate for 24 hours to reach 80% confluency. SINV was treated with BTE at various concentrations (0.7mM, and 1.4mM) and incubated for 1h at room temperature. The BTE treated virus was added to Vero cells at an MOI of 1 and allowed to adsorb for one hour. Unabsorbed virus was removed, and replaced with DMEM. The infected cells were incubated at 37°C and 5%CO₂ for 48 hours. Images were taken using an inverted light microscope 48 hours post infection.

- **MTS Antiviral Assay:**

Vero cells were seeded into a 96 well plate and incubated for 24 hours to reach 80% confluency. One hundred microliters of Sindbis virus stock was treated with various concentrations of BTE (0.7mM and 1.4mM). After 1h of exposure to the BTE at room temperature, 100μL of the BTE treated virus was added to Vero cells in individual wells. Cells were infected for 1h, with rocking every 15 minutes. Unabsorbed virus was removed and replaced with DMEM. Forty-eight hours post-infection, 20μL MTS reagent (Promega, Madison, WI) was added to each well. After a one hour incubation, absorbance was recorded at 450nm.

- **Viral ToxGlo**

Vero Cells were seeded into a 96 well plate and incubated for 24 hours to reach 80% confluency. One hundred microliters of Sindbis virus stock was combined

with various concentrations of BTE (0.7mM and 1.4mM). After one hour of exposure to the BTE at room temperature, 100 μ L of the BTE treated virus was added to Vero cells in individual wells. Cells were infected for 1h, with rocking every 15 minutes. Unabsorbed virus was removed and replaced with DMEM. Forty-eight hours post-infection, 100 μ L of ToxGlo (Promega, Madison, WI) reagent was added to Vero cells in each well. After a one hour incubation, relative luminescence units (RLU) were measured and recorded using a luminometer.

Results

Determining titer using plaque assay

Plaque assay was performed to determine the viral titer. Plaques on duplicate wells of the 10^{-4} dilution were counted, and their average was used in the viral titer calculation. The titer was calculated using the following equation:

$$\text{Titer (pfu/mL)} = (\# \text{ pfu} / \text{volume used in } \mu\text{L}) \times (10^3 \mu\text{l/mL}) \times (\text{dilution factor})$$

$$\text{Titer (pfu/mL)} = (9/500\mu\text{L}) \times (10^3 \mu\text{l/mL}) \times 10^4$$

$$1.8 \times 10^5 \text{ pfu/mL}$$

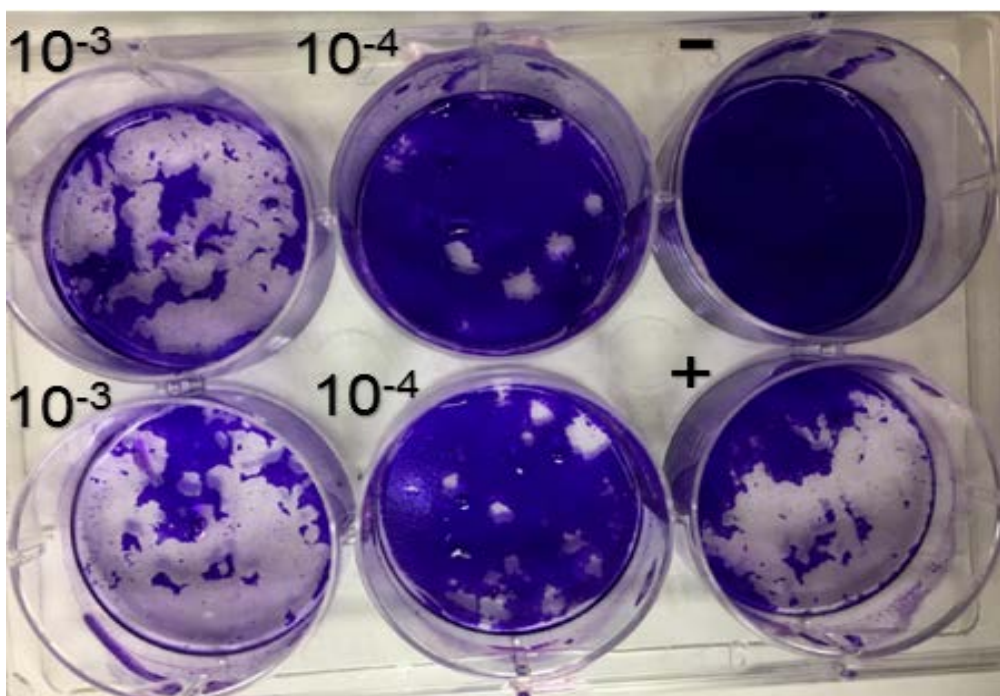


Figure 1: Plaque assay to calculate viral titer. Plaques on the 10^{-4} dilution were counted to calculate viral titer.

SINV induced cytopathic effects were reduced by treatment with 0.7mM BTE and 1.4mM BTE.

In order to determine if BTE could reduce cytopathic effects in SINV infected cells, SINV stocks were treated with BTE at 0.7mM and 1.4mM concentrations for one

hour prior to infection. Vero cells were then incubated with DMEM (negative control), or infected with SINV (positive control) or BTE treated SINV (Figure 2). Cytopathic effects included rounding and lifting of cells from the monolayer. Morphological changes were assessed by comparison of the BTE treated virions to the negative and positive controls. The negative control shows a healthy monolayer of cells (Figure 2a). The positive control shows a complete lifting of the cell monolayer along with full rounding of each cell, as a result of viral infection (Figure 2b). Cytopathic effects were substantially reduced by pretreatment of SINV with 1.4mM BTE (Figure 2c). Most of the cell monolayer is still visible, with only a few lifted cells, suggesting substantial antiviral activity at this concentration. For 0.7mM BTE, reduction of cytopathic effects is less apparent (Figure 2d). Many cells from the monolayer are lifted, but still to a lesser extent than the SINV infected cells, which is an indication of antiviral activity. Overall, treatment of SINV with 0.7mM and 1.4mM BTE concentrations demonstrate the ability of BTE to inhibit SINV infection in cultured cells, suggested by the reduction of SINV induced cytopathic effects. Antiviral properties of BTE are reduced with decreasing concentrations.

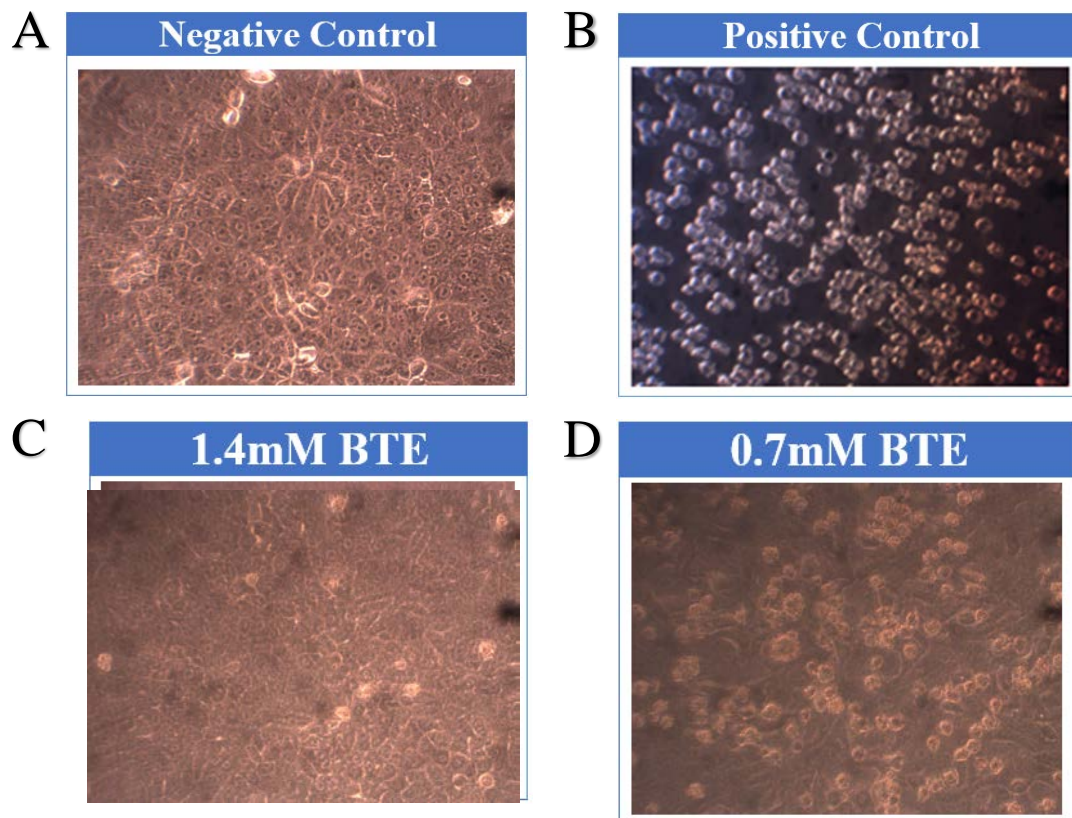


Figure 2: Reduction of Cytopathic effects by BTE pretreatment of SINV in Vero cells. 1a: Healthy monolayer of vero cells. 1b: Vero cells infected with SINV. 1c: Vero cells infected with BTE (1.4mM concentration) treated SINV. 1d: Vero cells infected with BTE (0.7mM concentration) treated SINV. All images were taken 48 hours post infection.

Concentrations of 0.7mM BTE and 1.4mM BTE inhibit SINV infection

The antiviral activity of BTE was measured using the MTS assay. This assay is a method of determining cell proliferation. Viable cells convert the MTS tetrazolium compound to formazan. The absorbance readings directly correlate to the amount of formazan present, which correlates to the number of viable cells present. Successful viral infection leads to a depletion of ATP, which results in lower absorbance readings and lower cell viability. Percent cell viability was calculated as a percentage relative to the negative control absorbance readings from DMEM and cells only wells. SINV exposure

to 0.7mM or 1.4mM BTE prior to infection resulted in higher cell viability than SINV infection with no treatment (Figure 3). Percent viability of SINV infected cells was 44.9 %. Pretreatment with 1.4mM BTE lead to a percent viability of 99.8%, supporting that BTE at this concentration displays antiviral activity. The 0.7mM BTE pretreatment resulted in a decreased cell viability of 70.25%. This suggests that the inhibition of SINV infection is concentration dependent. Percent inhibition was calculated via a comparison of absorbance readings from uninfected Vero cells, SINV stock infected, and BTE treated SINV infected cells. The equation used is seen below (Figure 4). Viral infection was inhibited 99% by 1.4mM BTE, while 0.7mM BTE showed a 46% inhibition (Figure 5). Inhibition of viral infection is observed in a concentration dependent manner. Treatment of SINV virions with 1.4mM BTE shows a percent inhibition comparable to the negative control (media + cells). This may be due to a great amount of antiviral activity. Treatment of SINV virions with 0.7mM BTE also inhibits viral infection, but less effectively.

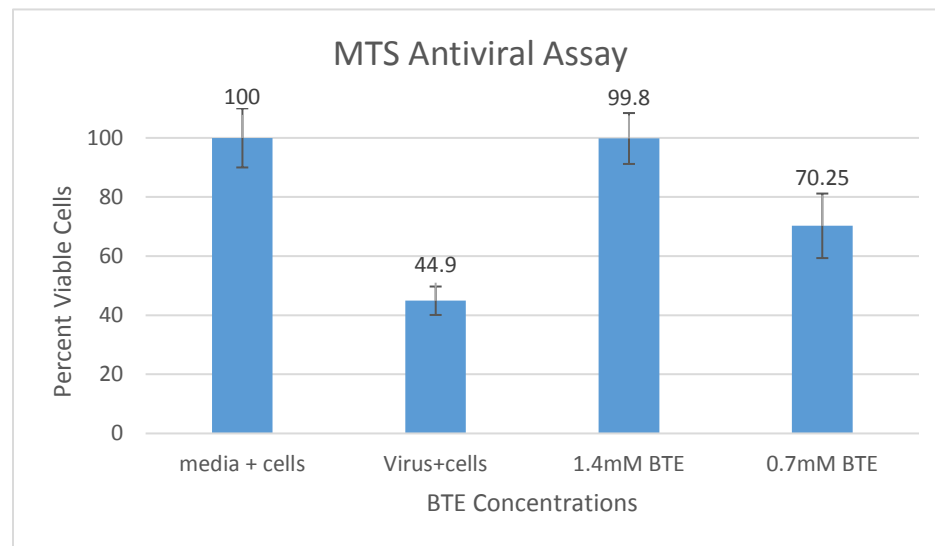


Figure 3: MTS antiviral assay results for SINV treated with BTE in Vero cells. The results indicate the percentage of viable cells. Pretreatment of SINV with 1.4mM and 0.7mM BTE resulted in higher cell viability than non-treated SINV, suggesting antiviral activity

$$\frac{(\text{Experimental well} - \text{virus only})}{(\text{Negative Control} - \text{virus only})}$$

Figure 4: Antiviral equation

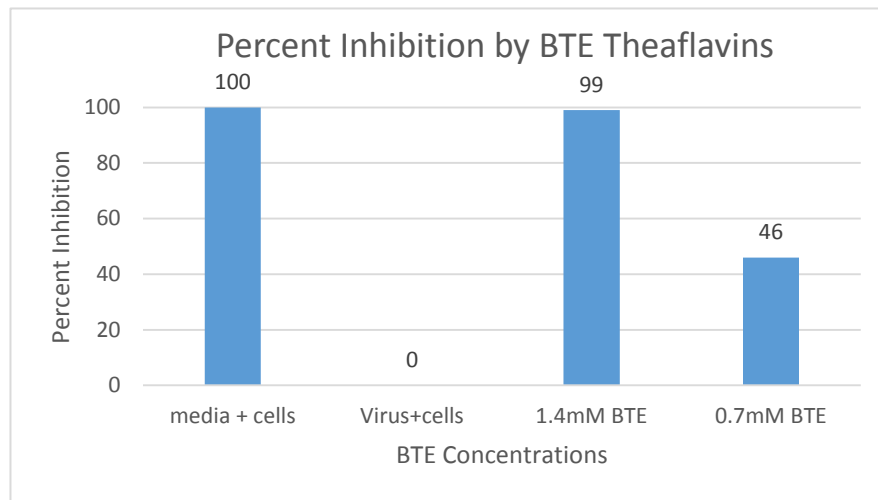


Figure 5: Percent inhibition of SINV infection by BTE in Vero cell. Results indicate the percent inhibition of SINV infection. Pretreatment with 1.4mM BTE resulted in 99% inhibition, while 0.7mM had a 46% inhibition of viral infection.

Viral ToxGlo Assay supports that both 0.7mM BTE and 1.4Mm BTE exhibit antiviral activity against SINV

A viral ToxGlo assay was conducted to provide a more accurate quantitative measure of viral inhibition. Cells were either incubated with DMEM, infected with SINV, or infected with BTE treated SINV. The assay measures the levels of ATP in cell supernatants. Relative light unit (RLU) values, correlate with the amount of ATP present, which correlates with the number of viable cells. The results of the Viral ToxGlo assay showed that infection of Vero cells with SINV treated with 1.4mM BTE has RLU values comparable to the negative control (media + cells), and much greater than the positive

control (virus + cells) (Figure 6). This suggests that viral infection was greatly inhibited. Antiviral activity is exhibited by SINV treated with 0.7mM BTE to a lesser extent, consistent with microscopic images of CPE and MTS assay results. Percent inhibition was calculated using the equation in Figure 3. SINV treated with 1.4mM BTE had an inhibition of 97.5%, while SINV treated with 0.7mM BTE had an inhibition of 76% (Figure 7). Again antiviral activity decreases with concentration. The concentration dependent antiviral activity observed in the ToxGlo correlates with microscopic images of CPE and with MTS assay results.

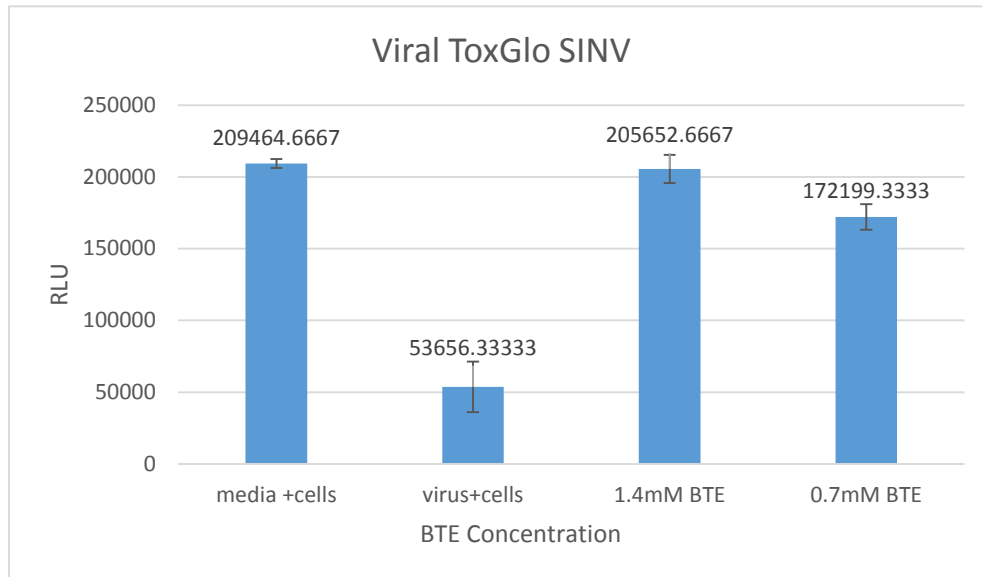


Figure 6: Viral ToxGlo assay for BTE treated SINV in Vero cells. The graph shows measurement of RLUs. RLU values directly correlate to the amount of viable cells. Pretreatment of SINV with 1.4mM BTE resulted in RLU values similar to the negative control (media + cells). Pretreatment with 0.7mM BTE also resulted in RLU values comparable to the negative control. This suggests BTE exhibits antiviral activity at the tested concentrations.

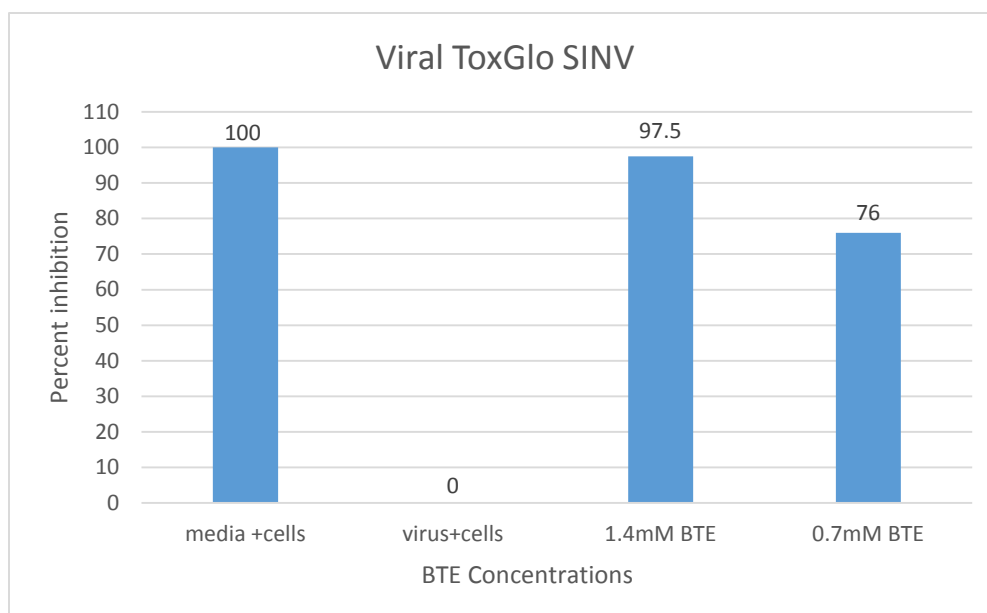


Figure 7: Viral ToxGlo percent inhibition. The graph shows percent inhibition of viral infection determined by ToxGlo. Percent inhibitions for 0.7mM and 1.4mM BTE are 76% and 97.5%, respectively.

Discussion

Studies suggest that consumption of tea may reduce the prevalence of cardiovascular disease and cancer (Yang and Landau 2000). Black tea makes up 80% of all the tea consumed worldwide. The polyphenol content in tea is considered the source of the bioactivity attributed to the beverage. Tea polyphenols are also natural antioxidants and antiviral molecules. Theaflavins, the black tea polyphenol, has consistently been shown to have antiviral activity for a broad range of viruses such as HSV-1, HIV-1, Caliciviruses, and Influenza A and B (Oliveira et al., 2015; Yang et al., 2011; Yang et al., 2015; Ohba et al., 2017).

Sindbis (SINV) is an arthropod-borne old world Alphavirus that causes fever, malaise, and arthralgia. SINV was used in this study as the model Alphavirus. Cases of SINV have not been reported in the Americas, but recently a more pathogenic old world

alphavirus Chikungunya has been detected in the Caribbean and the United States. In 2015, it was reported that epigallocatechin-3- gallate (EGCG), the major green tea polyphenol, inhibited Chikungunya infection *in vitro* (Weber et al., 2015). Considering that theaflavins are derived from green tea catechins, we hypothesized that theaflavins would also exhibit antiviral activity against Alphaviruses. This study is the first demonstration of the antiviral activity of black tea extract containing theaflavins against an Alphavirus.

Virus inhibition was microscopically assessed through an inverted light microscope, but also quantitatively studied using MTS and ToxGlo cell proliferation assays. A previous study showed that BTE is not cytotoxic to Vero cells at concentrations up to 14mM (Cantatore et al., 2013). In this study, SINV virions were treated with 0.7mM and 1.4mM BTE prior to infection of Vero cells. Light microscope images showed that cytopathic effects were reduced almost entirely by treatment of SINV with 1.4mM concentration of BTE prior to infection, consistent with previous findings (Yang et al., 2011; Zu et al., 2011). Cytopathic effects were also reduced by 0.7mM BTE, but as the concentration of BTE decreased, so did antiviral activity. Treatment of cells with BTE prior to SINV infection did not reduce CPE (Data not shown).

Percent inhibition values were calculated using the MTS and ToxGlo proliferation assays. For both MTS and ToxGlo assays the percent inhibition was greater than 95% for SINV treated with 1.4mM BTE. Antiviral activity of BTE, specifically for SINV virions treated with a concentration of 1.4mM, has previously been reported (Cantatore et al., 2013). For virions treated with 0.7mM BTE the percent inhibition values for MTS and ToxGlo assays were 47% and 76%, respectively. The difference in these values may be

due to the nature of both assays. ToxGlo is a more accurate and reliable method of measuring viral induced cytopathic effects, compared to the MTS assay. The concentration dependent antiviral activity of BTE in this study is consistent with previous studies (Zu et al., 2011; Ohba et al., 2017). Throughout this study, SINV stocks were exposed to BTE prior to infection of Vero cells. This suggests that BTE theaflavins act on viral proteins inhibiting infection by preventing adsorption to host cells, consistent with previous studies on the antiviral mechanisms of theaflavins (Liu et al., 2005 and Yang et al., 2012). SINV glycoprotein E2 is responsible for interacting with the surface of host cells, facilitating receptor mediated endocytosis. It is probable that theaflavins disrupt the interaction of E2 with the host cell, resulting in poor attachment, and eventually no viral infection. A study showed that specific amino acid mutations in E2 can result in decreased titers, and even no detectable infectious particles (Snyder et al., 2012). This shows how essential E2 is for viral entry, and why this glycoprotein would be an ideal target for theaflavins.

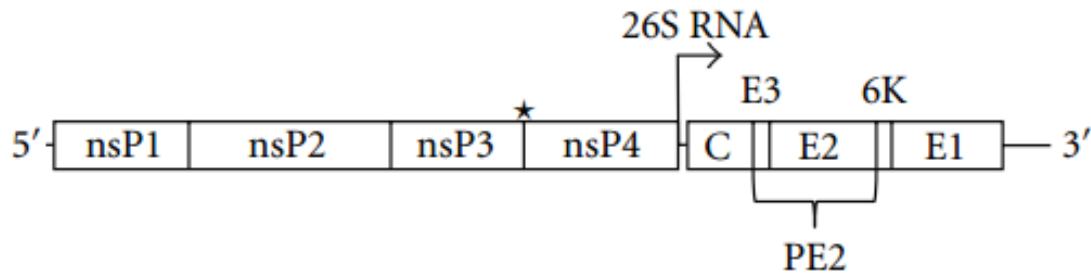
This study suggests that black tea extract (BTE; $\geq 80\%$ theaflavin) has antiviral activity against SINV. The antiviral activity of BTE against SINV suggests that other Alphaviruses may also be inhibited by theaflavins. The cost efficiency of BTE makes it a potential candidate to be further studied and potentially developed as an anti-alphavirus drug.

Conclusions

Overall, black tea theaflavins were shown to have antiviral activity against SINV, an Alphavirus, *in vitro*. Treatment of virions with 1.4mM BTE concentration exhibited a

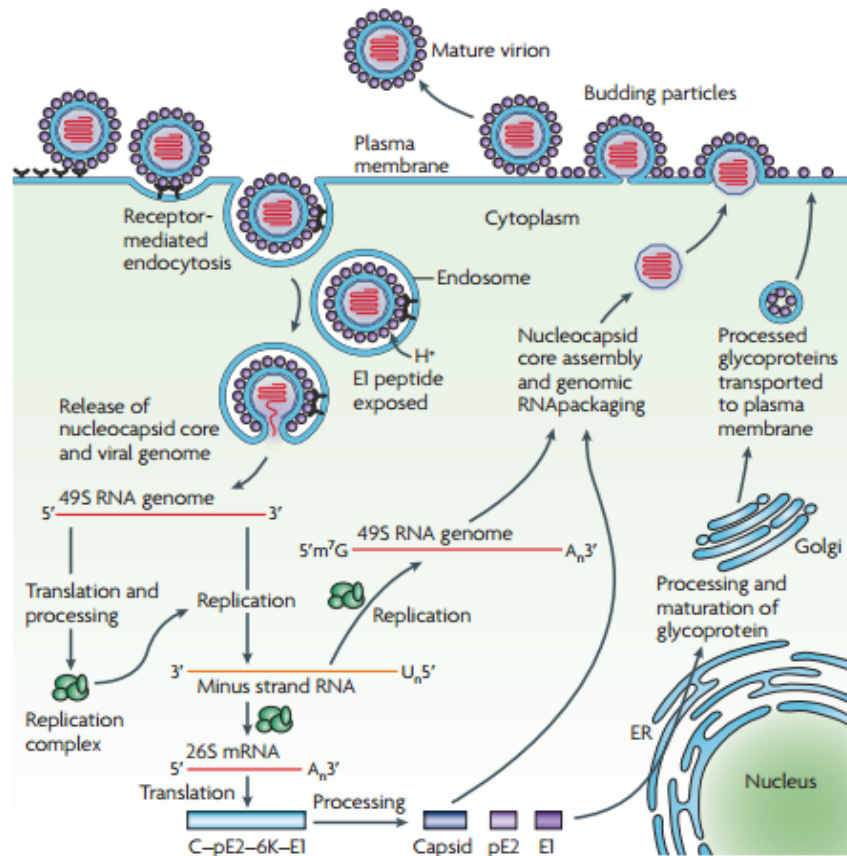
substantial antiviral activity, much greater than virions treated with 0.7mM BTE. The antiviral properties of black tea polyphenols may be a result of disrupting viral glycoproteins from interacting with host cell receptors. Cytopathic effects images, MTS and ToxGlo assay results suggest that 1.4mM BTE inhibits SINV infection by over 95%. These results indicate that the antiviral activities of black tea extract ($\geq 80\%$ theaflavins) warrant further study.

APPENDIX Supplemental Figures



Supplemental Figure 1: Sindbis virus genome diagram. The genome is composed of two open reading frames, encoding 4 non-structural and 5 structural proteins (C, E3, E2, E1).

Box 2 | The alphavirus life cycle



Supplemental Figure 2. The Alphavirus Life Cycle. Virions enter via receptor-mediated endocytosis, and are then found in endosomes. As the pH inside endosomes drop, the nucleocapsid core and viral genome are released. The replication complex composed of non-structural proteins 1-4 is formed, allowing for the replication of both genomic (49S) and subgenomic (27S) RNA. Genomic RNA is packaged inside a nucleocapsid, while glycoproteins are processed and transported to the plasma membrane. Lastly, newly formed virions exit the cell.

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